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Activation of human T cell leukemia virus type 1 LTR promoter and cellular promoter elements by T cell receptor signaling and HTLV-1 Tax expression

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Abstract

Human T cell leukemia virus 1 (HTLV-1) gene expression is regulated by both the viral Tax protein and by cellular transcriptional factors. We have previously shown that immune activation stimuli such as phorbol esters (PMA) and phytohemagglutinin (PHA) cooperate with HTLV-1 Tax expression to enhance HTLV-1 gene expression in infected T cells through increased activity of the HTLV-1 LTR. We now extend these studies to demonstrate roles for the T cell receptor complex, Lck, and Ras molecules in the coactivation of the HTLV-1 LTR by Tax and T cell activation stimuli. We also observe coactivation of Tax-responsive cellular promoter elements containing NF-κB and serum response factor (SRF) binding sites by Tax and T cell activation stimuli. These results suggest a model whereby T cell receptor stimulation and Tax expression coactivate HTLV-1 gene expression and cellular gene expression, enhancing activation of latent HTLV-1 and expression of cellular genes involved in disease pathogenesis.

Keywords: HTLV-1; LTR; Latency; Reactivation; T cell activation; Lck; Ras; SRF; NF-κB

Introduction

HTLV-1 was the first identified human retrovirus (Poiesz et al., 1980; Yoshida et al., 1982) and was shown to be the causative agent of adult T cell leukemia/lymphoma (ATL) (Blattner et al., 1982; Hinuma et al., 1981) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al., 1985; Osame et al., 1986). HTLV-1 infection is endemic in a number of parts of the world, including the southern islands of Japan, Brazil, and the Caribbean, and parts of central Africa. HTLV-1 infection is also seen in the southern United States, as well as in immigrants, particularly from the Caribbean (Manns et al., 1999). Infection with HTLV-1 is associated with a diverse set of potential clinical outcomes ranging from asymptomatic infection, to the development of ATL, HAM/TSP, or a variety of autoimmune syndromes (reviewed in Höllsberg and Hafler, 1993; Uchiyama, 1997). The molecular basis for the development of each of these different disorders is unclear, but is likely to be dependent on complex host–virus interactions, as the overwhelming majority of HTLV-1-infected individuals remain asymptomatic, and the likelihood of developing a particular disease is, in part, determined by the routes and timing of viral infection. For example, ATL develops in

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less than 3–5% of infected individuals, often those infected in childhood, with a clinical latency of up to 20–60 years (Mortreux et al., 2003). In contrast, HAM/TSP may develop in up to 3–5% of individuals infected later in life, with shorter clinical latency periods.

HTLV-1 infects predominantly human CD4+, CD45RO+ T cells in vivo (reviewed in Höllsberg, 1999). Infection of CD4+ T cells in vitro results in immortalization and ultimately growth of IL-2-independent T cells (Popovic et al., 1983). This in vitro transformation provides an interesting analogy to the proliferation of oligoclonal and ultimately monoclonal populations of T cells seen in association with HTLV-1-induced diseases. The detailed mechanisms responsible for this immortalization are not yet known; however, they clearly involve the activities of the HTLV-1 transcriptional transactivator, Tax (reviewed in Franchini et al., 2003; Yoshida, 2001). In addition to potently activating transcription of the HTLV-1 long terminal repeat (LTR) through the LTR Tax response elements (TREs), Tax activates transcription of a large array of cellular genes, including genes likely to be directly involved in T cell transformation (reviewed in Azran et al., 2004). Tax also affects the functions of cell cycle regulatory genes (Jeang, 2001). The ability of HTLV-1 to induce proliferation of infected cells undoubtedly plays a role in pathogenesis of the HTLV-1-associated disorders. Nevertheless, there are clearly fundamental differences between the rapid transformation of T cells in vitro by HTLV-1 and the long-term in vivo development of ATL, including differences in HTLV-1 gene expression in the two different settings.

Viral latency and reactivation is a common feature of retroviral infections and may contribute to viral persistence and disease pathogenesis. In human immunodeficiency virus type 1 (HIV-1) infection, viral latency has been well characterized both molecularly and in the effects that latently infected cells have on viral persistence and antiviral therapy (Persaud et al., 2003). Latency in HTLV-1 infection has been less characterized, although evidence for in vivo latency and identification of in vitro cellular models of latent infection have both been described. In vivo, it is clear that HTLV-1 infection can result in either silent or productive infection of individual T cells. For example, in HAM/TSP patients, up to 15–18% of T cells in peripheral blood were HTLV-1 infected, although only a few cells (perhaps 1/5000) expressed high levels of HTLV-1 Tax in vivo (Höllsberg, 1999). In contrast, HTLV-1 Tax expression was rapidly induced following culture of PBMCs from infected patients in the presence of PHA and IL-2, suggesting that many cells in vivo may be latently infected and contain inducible proviruses (Kinoshita et al., 1989). Not all latently infected cells contain inducible HTLV-1 genomes (Richardson et al., 1997) and many contain proviruses that have sustained various inactivating mutations (Rose et al., 2000); however, latently infected cell clones have been described that clearly exhibited inducible HTLV-1 gene expression in culture (Richardson et al., 1997). In addition to proviral mutational inactivation, transcriptional repression of HTLV-1 LTR function has been proposed to contribute to viral latency. This may be due to either *cis*-acting epigenetic events such as methylation of HTLV-1 proviral DNA (Saggioro et al., 1991; Takeda et al., 2004) and recruitment of histone deacetylases to the LTR promoter (Ego et al., 2002; Lemasson et al., 2004; Lu et al., 2004), or effects of cellular transcription factors such as NF-κB-2 p100-mediated inhibition of Tax (Beraud et al., 1994), ICER-mediated inhibition of Tax activation through CREB/ATF (Newbound et al., 2000), and the possible inhibitory effects of CREB/ATF complexes binding to the LTR R region (Xu et al., 1994).

Several groups have begun to examine the types of stimuli that may reactivate expression of latent HTLV-1. Studies from our laboratory (Lin et al., 1998) have identified HTLV-1-infected T cell lines that express only very low levels of HTLV-1 RNA and proteins, but which can be induced to express much higher levels of HTLV-1 gene products by treatment with T cell activation stimuli, such as phytohemagglutinin (PHA) and phorbol esters (PMA). Although the proviruses in these cells are not completely silent, the ability to markedly activate very limited basal HTLV-1 gene and protein expression suggests that these cells may be a model of HTLV-1 latency and reactivation. These studies suggested that these activation stimuli cooperate with HTLV-1 Tax to synergistically activate HTLV gene expression and HTLV-1 LTR-mediated transcription. Similarly, Dumais et al. have demonstrated that the combination of T cell receptor and CD28 stimulation with prostaglandin E_2 treatment results in strong activation of the HTLV-1 LTR and integrated HTLV-1 provirus (Dumais et al., 2003). These authors have further implicated a role for tyrosine phosphorylation in this activation (Langlois et al., 2004). Lairmore et al. demonstrated that anti-CD2 antibody activated HTLV-1 transcription from infected cells (Guyot et al., 1997), and have also demonstrated that cellular stresses, such as those induced by heat stress and sodium arsenite enhanced HTLV-1 gene expression in chronically infected cell lines (Andrews et al., 1995). This activity may be independent of Tax and was mediated through effects on formation of RNA polymerase complexes at the promoter (Andrews et al., 1997). Cellular stresses such as UV light (Okada and Jeang, 2002) and other DNA damaging agents (Torgeman et al., 2001a) have also been shown to activate integrated LTRs. Others have also shown activation of the HTLV-1 LTR by phorbol esters in non-T cells (Jeang et al., 1988; Torgeman et al., 2001b), and cooperative effects of Tax and signal transduction pathways involving Ras have also been demonstrated (Pozzatti et al., 1990). Overall, these studies strongly suggest that immune activation, cellular stresses, and intracellular signal transduction pathways may activate the transcription of integrated HTLV-1 proviruses and therefore may activate expression of latent HTLV-1. In this study, we demonstrate that T cell activation signals mediated through Lck and Ras

contribute to the coactivation of the HTLV-1 LTR with Tax and that cellular promoters responsive to Tax may exhibit similar coactivation by T cell activation stimuli. These observations begin to define pathways and targets that may mediate activation of latent HTLV-1 in vivo and suggest roles that T cell activation may play in the pathogenesis of HTLV-1-induced disease.

Results

Activation of HTLV-1 gene expression through T cell receptor stimulation

Previously, we demonstrated that treatment of T cells chronically infected with HTLV-1 with PHA or PMA resulted in induction of expression of HTLV-1 RNA and proteins and enhanced Tax transactivation of the HTLV-1 LTR in stimulated cells (Lin et al., 1998). These results suggested that immune activation stimuli, working through the T cell receptor signaling pathway, may activate integrated HTLV-1 proviruses. FS cells, an HTLV-1-infected T cell line derived from a HAM/TSP patient (Dezzutti et al., 1993), normally express low or non-detectable levels of the HTLV-1 Tax protein. We used two different approaches to directly examine the effect of T cell receptor signaling on HTLV-1 gene expression in FS cells. In the first approach (Fig. 1A, upper panel), FS cells were incubated with anti-CD3 antibody to induce T cell receptor crosslinking. This experiment used a well-established system for inducing antibody crosslinking of target cell surface receptors, in which the antibody under study is bound to the surface of irradiated murine L-thymidine kinase-negative cells which express the human Fc gamma RII receptor (CDw32 L cells) (Peltz et al., 1988). The CDw32 L cells were incubated first with OKT3 antibody (anti-CD3 antisera) to coat their surface with the anti-CD3 antibody and then cocultivated with FS cells to allow binding of the OKT3 antibody to CD3

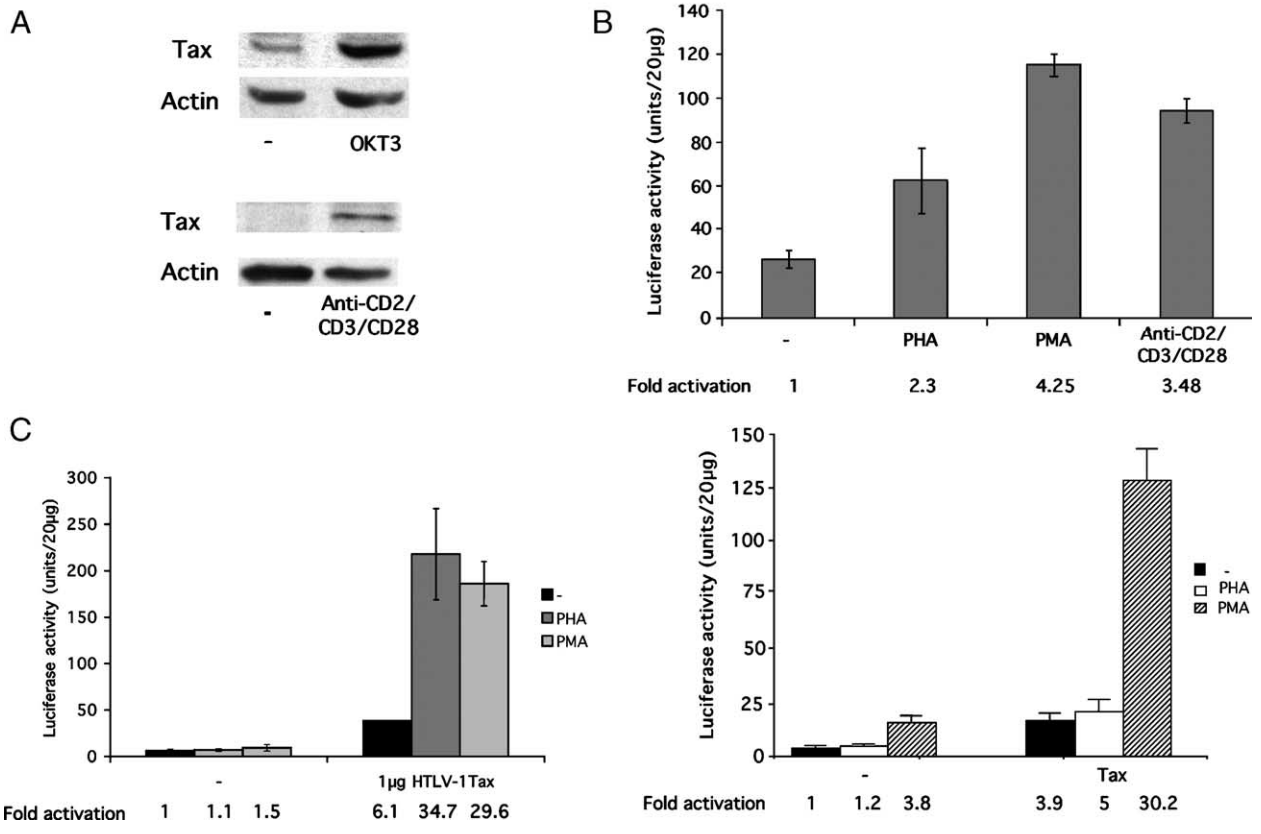

Fig. 1. Activation of HTLV-1 gene expression through T cell receptor stimulation. (A) Activation of Tax protein expression in FS cells subjected to T cell receptor activation. (Upper panel) Whole-cell extracts were prepared from FS cells, which were co-cultivated for 48 h with mouse CDw32 L cells pre-incubated with OKT3 antibody. Western blot analysis was performed using antisera directed against the HTLV-1 Tax protein. Anti-actin antibody was used as control. (Lower panel) FS cells were incubated with anti-CD2/CD3/CD28-loaded anti-Biotin MACSiBead particles for 48 h. Western blot analysis was performed on cell lysates using anti-sera directed against the HTLV-1 Tax protein. Anti-actin antibody was used as control. (B) T cell activation signals activate HTLV-1 LTR-directed gene expression in FS cells. The pHTLV-1Luc plasmid was transfected into FS cells. PHA, PMA or anti-CD2/CD3/Cd28 beads were added 24 h after transfection. Luciferase activity was assayed 40 h after transfection. Average luciferase activities with standard deviations (error bars) from triplicate transfections are shown and the fold activation compared to untreated cells is indicated. (C) Role of T cell receptor in HTLV-1 LTR activation. The pHTLV1 Luc plasmid was cotransfected into Jurkat cells (left panel) or J.RT3 cells (right panel), a CD3-negative Jurkat cell line, along with the Tax expression plasmid, with or without treatment with PHA or PMA. Reporter assays were performed as described above. Average luciferase activities with standard deviations (error bars) from triplicate transfections are shown and the fold activation compared to the luciferase value from untreated cells in the absence of Tax is indicated.

antigen on the surface of the FS cells. In this experiment, anti-CD3 antibody induced expression of the HTLV-1 Tax protein in FS cells approximately 4-fold as compared with the very low basal level in untreated cells. In the second approach, we incubated FS cells with beads pre-coated with anti-CD2, anti-CD3, and anti-CD28 antibodies to induce T cell receptor crosslinking and T cell activation. Untreated and treated FS cells were lysed and examined for HTLV-1 Tax gene expression by Western blot analysis. As shown in Fig. 1A lower panel, treatment of FS cells with the anti-CD2/CD3/CD28 coated beads led to a marked induction of HTLV-1 Tax protein expression from essentially undetectable levels to easily detectable Tax protein. Levels of control actin protein actually slightly decreased following treatment. These results confirm that, as previously suggested by PHA treatment, direct T cell receptor stimulation is capable of inducing Tax expression in these latently infected cells.

Our previous studies suggested that the PHA and PMA induction of HTLV-1 protein expression was due to enhanced transcription of the HTLV-1 LTR. PHA treatment resulted in increased HTLV-1 RNA expression in FS cells, and in increased Tax-mediated transactivation of the HTLV-1 LTR in transiently transfected Jurkat T cells (Lin et al., 1998 and Fig. 1C, left). In order to confirm that LTR activation of transfected LTR-reporter plasmids transiently transfected into Jurkat cells models the LTR activation seen in the FS cells, we transfected the HTLV-1 LTR-luciferase plasmid directly into FS cells and subjected the transfected cells to treatment with PHA, PMA, or incubation with the anti-CD2/CD3/CD28 antibody-coated beads. As shown in Fig. 1B, PHA, PMA, and antibody crosslinking of T cell surface markers all resulted in significant activation of the transfected HTLV-1 LTR in the FS cells, leading to a 2.3-, 4.2-, and 3.5-fold activation of reporter gene expression, respectively. Thus, cotransfection of T cells with the HTLV-1 LTR-luciferase reporter plasmid and a Tax-expressing plasmid, followed by treatment of transfected cells with T cell activation stimuli, appears to mimic the activation of the endogenous HTLV-1 LTRs and endogenous Tax expression present in FS cells.

In order to further understand the mechanisms responsible for HTLV-1 LTR activation by T cell activation stimuli, we have employed transient transfection of Jurkat T cell lines in which components of the T cell activation pathway are mutated. To further assess the role of CD3 on PHA/Tax-mediated coactivation of the HTLV-1 LTR, we transfected a CD3-negative mutant of Jurkat T cells (J.RT3 cells; Ohashi et al., 1985) with the HTLV-1 LTR reporter plasmid, with or without the LTR-Tax expression plasmid (Fig. 1C). Transfected cells were either untreated or treated with PHA or PMA. As a control, the results of transfection of wild-type Jurkat cells are shown in the left panel for comparison and demonstrate coactivation of the HTLV-1 LTR by Tax transfection and PHA or PMA treatment (35- and 30-fold, respectively), but only low levels of direct activation of the LTR by these stimuli (1.1- and 1.5-fold) similar to our previous observations (Lin et al., 1998). As shown in Fig. 1C right panel, in J.RT3 cells, PHA no longer enhanced Tax-mediated HTLV-1 promoter activity, while PMA still cooperated with Tax to activate HTLV-1 LTR-directed gene expression. These data suggest that the CD3/TCR complex is required for coactivation of HTLV-1 expression in response to PHA stimulation; however, it is not required for activation by phorbol esters, which act on protein kinase C isoforms downstream of TCR crosslinking in the process of T cell activation.

Role of intracellular signaling molecules in TCR-mediated coactivation of the HTLV-1 LTR

In our previous study, we tested the effects of inhibitors of steps in the T cell activation signal transduction pathway on the activation of HTLV-1 gene expression and of the HTLV-1 LTR by Tax/PHA or Tax/PMA. In those studies, a tyrosine kinase inhibitor potently blocked both PHA- and PMA-induced activation, while a PKC inhibitor blocked activation by PMA but not PHA. Lck is an important tyrosine kinase in T cell receptor-mediated activation, which plays a role following TCR crosslinking in assembling the signaling machinery at the membrane, and activating a series of downstream kinases (reviewed in Chan et al., 1994; Singer and Koretzky, 2002). JCaM.1 cells are mutant Jurkat T cells, defective in Lck tyrosine kinase function (Gupta et al., 1994). We employed JCaM.1 cells to test whether Lck played a role in the activation of the HTLV-1 LTR following PHA treatment. JCaM.1 cells were transiently transfected with the HTLV-1 LTR luciferase plasmid, with or without an HTLV-1 LTR-Tax expression plasmid. Transfected cells were treated with PHA or PHA. While Tax transfection and PMA treatment still resulted in marked synergistic activation of the HTLV-1 LTR in JCaM.1 cells, PHA plus Tax only minimally further activated HTLV-1 LTR expression beyond that observed with Tax alone (Fig. 2). Transfection of an Lck expression plasmid back into JCaM.1 cells restored significant levels of coactivation by Tax and PHA, although these levels remained lower than those observed in wild-type cells (Fig. 2). Overexpression of Lck, even in the absence of PHA, also modestly enhanced Tax activation of the LTR. These data suggest that Lck plays a significant role in transduction of the PHA/TCR signal for activation of HTLV-1 expression, and itself may enhance Tax transactivation.

Ras proteins play an important role in signal transduction mediated by the T cell receptor following antigenic stimulation (reviewed in Cantrell, 2003). As an initial test of the possible role of Ras proteins in signal transduction pathways leading to synergistic activation of the HTLV-1 LTR by T cell activation stimuli and Tax, we assayed the effects of activated Ras on HTLV-1 LTR expression. Jurkat cells were cotransfected with an expression plasmid encoding the oncogenically activated form of v-H-Ras. As shown in Fig. 3A, while Ras alone has little effect on HTLV-1 LTR function in these cells, Ras plus Tax cotransfection results in

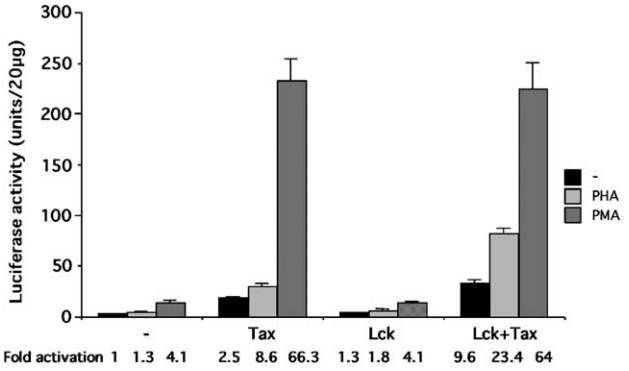

Fig. 2. Role of Lck in activation of the HTLV-1 LTR by PHA/Tax or PMA/Tax in T cells. The pHTLV-1 Luc plasmid was cotransfected into JCaM.1, an Lck-defective Jurkat cell line, along with the Tax expression plasmid, with or without cotransfection of an Lck expression plasmid (pcDNA-Lck). Stimuli were added 24 h after transfection. Luciferase activity was assayed 40 h after transfection. Average luciferase activities with standard deviations (error bars) from triplicate transfections are shown and the fold activation compared to untreated cells is indicated.

marked synergistic activation of the HTLV-1 LTR, equivalent to that seen with Tax plus PHA or PMA. This is particularly interesting in view of the reported co-transforming activities of Ras and Tax (Pozzatti et al., 1990). In the presence of transfected Ras, PHA or PMA treatment had little additional effect on Tax transactivation of the HTLV-1 LTR. These data suggest that Ras can potently cooperate with Tax in activation of Tax-responsive genes. The fact that Ras cooperation with Tax results in a coactivation similar to that observed with PHA or PMA and does not further enhance the PHA/Tax or PMA/Tax activation is consistent with Ras being in the same signal transduction pathway as PHA or PMA treatment in inducing LTR activation, as might be expected based on its known roles in TCR-mediated signal transduction. As shown in Fig. 3B, conversely, cotransfection of a dominant-negative H-Ras (Feig and Cooper, 1988) resulted in a significant reduction of Tax/PHA or Tax/PMA coactivation. Interestingly, the dominant-negative Ras also reduced Tax-mediated transactivation approximately 2-fold. It is therefore difficult to specifically identify an effect of Ras in the PHA or PMA coactivation pathway that is distinguishable for the effects of Ras on Tax itself but this result raises the interesting possibility that Tax-mediated transactivation itself is enhanced by Ras-regulated pathways.

Effects of T cell activation stimuli on Tax activation of cellular promoter elements

In addition to regulating HTLV-1 gene expression, Tax has also been shown to alter cellular gene expression through a number of different transcriptional regulatory pathways (reviewed in Azran et al., 2004). These include activation of cellular promoters containing NF-κB binding sites, serum response elements, CREB/ATF family protein binding sites, and other transcription factors. These effects on cellular promoters are likely to play important roles in Tax-mediated transformation of T cells. We were interested in assessing whether Tax could also cooperate with PHA or PMA in the activation of promoters containing these transcription factor binding sites. Tax activation of promoters containing NF-κB sites likely plays a critical role in T cell transformation. We therefore tested whether a promoter directed by multimerized NF-κB binding sites derived from the IL-6 gene, a known Tax-responsive promoter (Muraoka et al., 1993), would exhibit increased activation by cotransfection with Tax and treatment with PHA or PMA. As shown in Fig. 4A, PHA or PMA stimulation alone activated the IL-6 κB promoter 7- to 8-fold, in the absence of Tax. HTLV-1 Tax activated the promoter approximately 8-fold. The combination of PHA or PMA with Tax cotransfection activated the IL6-κB promoter approximately 7- to 9-fold over that seen with Tax alone, or 65- to 80-fold over basal promoter activity. Thus, for the NF-κB-directed promoter, PMA and PHA exerted a similar effect on promoter activity in the presence or absence of

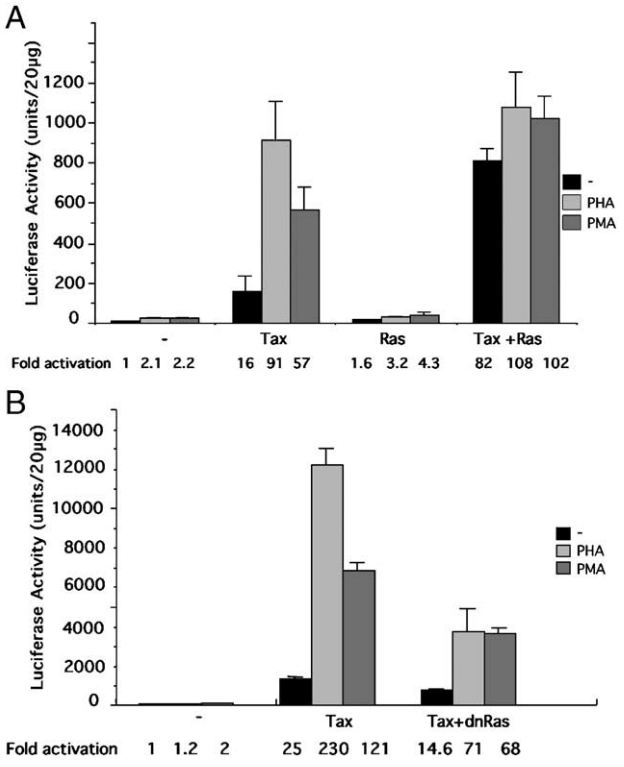

Fig. 3. Role of Ras in activation of the HTLV-1 LTR by PHA/Tax or PMA/Tax. (A) The pHTLV-1 Luc plasmid was cotransfected into Jurkat cells with the Tax expression plasmid and with an activated v-Ras expression vector (pCMV-vRas). Stimuli were added 24 h after transfection. Luciferase activity was assayed 40 h after transfection. (B) Effect of dominant-negative H-Ras on PHA/Tax or PMA/Tax coactivation. The pHTLV-1 Luc plasmid was cotransfected into Jurkat cells with the Tax expression plasmid (pHTLV-1 LTR-Tax) and a dominant-negative H-Ras expression vector (pMZH17N). Stimuli were added 24 h after transfection. Luciferase activity was assayed 40 h after transfection. Average luciferase activities with standard deviations (error bars) from triplicate transfections are shown and the fold activation compared to untreated cells is indicated.

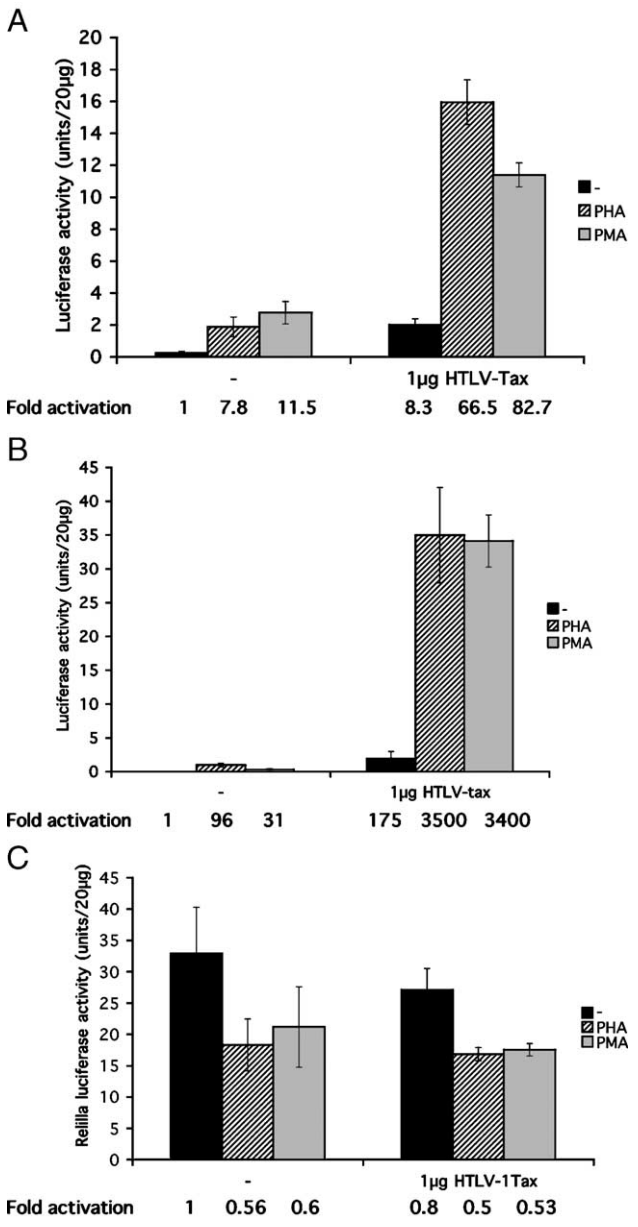

Fig. 4. Effects of T cell activation stimuli on Tax-mediated-activation of cellular promoter elements. (A) Activation of an NF-κB-responsive promoter by Tax/PHA and Tax/PMA. The pIL6κB-Luc reporter plasmid was transfected into Jurkat T cells, with or without cotransfection of the Tax expression plasmid. PHA and PMA were added at 24 h after transfection. Luciferase activity was assayed 40 h after transfection. (B) Activation of serum-responsive element containing promoter by Tax/PHA and Tax/PMA. The pSRE-Luc reporter plasmid was transfected into Jurkat T cells, with or without cotransfection of the Tax expressing plasmid. PHA and PMA were added at 24 h after transfection. Luciferase activity was assayed 40 h after transfection. (C) Activation of the HSV-TK promoter by Tax/PHA and Tax/PMA. A plasmid containing the Renilla luciferase reporter gene under the control of the HSV-TK promoter (pRL-TK) was transfected into Jurkat T cells, with or without cotransfection of the Tax-expressing plasmid. PHA and PMA were added at 24 h after transfection. Renilla Luciferase activity was assayed 40 h after transfection. Average luciferase activities with standard deviations (error bars) from triplicate transfections are shown and the fold activation compared to untreated cells is indicated.

Tax. The combination of the activating effects of Tax and PMA/PHA resulted in a marked increase in overall promoter activity, but not associated with the kind of synergistic promoter activation seen for the HTLV-1 LTR. The serum response elements (SREs) direct transcription of immediate early genes, such as c-*fos*, also likely to play important roles in Tax-mediated transformation. To assess whether PHA or PMA would cooperate with Tax in the activation of SRE-containing promoters, a reporter plasmid directed by multiple copies of SREs was transfected in Jurkat T cell, with HTLV-1 Tax cotransfection (Fig. 4B). Again, PHA and PMA treatment, as well as Tax cotransfection, each substantially increased the very low basal levels of SRE promoter activity, while the combination of Tax and PHA or PMA again resulted in markedly higher levels of SRE promoter activity. Tax cotransfection or PHA/PMA treatment did not activate the herpes simplex virus thymidine kinase (TK) promoter (Fig. 4C), suggesting the observed PHA/Tax or PMA/Tax coactivation is promoter-specific, dependent on basal Tax activation of target promoters, and is therefore not a general property of these stimuli.

Discussion

Our previous study (Lin et al., 1998) showed that the T cell activation stimuli, PHA and PMA, induced HTLV-1 gene expression from chronically infected T cell lines. This induction appeared to be mediated through synergistic activation of the HTLV-1 LTR by these agents in combination with the HTLV-1 Tax transactivator. In this study, we have further characterized the signal transduction pathways responsible for the induction of HTLV-1 transcription mediated by T cell activation stimuli and Tax. We have also demonstrated strong enhancement of cellular promoter elements through the combined effects of Tax and T cell activation stimuli. An important component of PHA-induced T cell activation is contributed through binding to, and crosslinking of, components of the CD3/T cell receptor complex. In our previous study, only the two cell lines expressing surface CD3 exhibited activation of HTLV-1 gene expression in response to PHA, suggesting that crosslinking of the CD3/TCR complex might be the first step in PHA-induced HTLV-1 activation. We extended this observation by demonstrating that activation of T cells by crosslinking CD3/TCR on the surface of HTLV-1-infected FS cells also resulted in enhanced Tax expression. Similarly, crosslinking of a combination of CD2, CD3, and CD28 also induced HTLV-1 gene expression in the FS cells. Furthermore, PHA no longer cooperated with HTLV-1 Tax in activation of the HTLV-1 in J.RT3 cells, a CD3-negative Jurkat T cell line, also consistent with the role for CD3 in the coactivation by PHA and Tax. Interestingly, a recent study has demonstrated potent activation of HTLV-1 LTR expression by a combination of crosslinking of CD3 and CD28, coupled with treatment with prostaglandin E_2

(Dumais et al., 2003). A weaker, but significant activation of both HTLV-1 LTR-luciferase plasmids, as well as of HTLV-1 p19 production from chronically infected cells induced by CD3 alone, was also observed in that study, consistent with the results that we have seen. Induction of HTLV-1 gene expression by CD3/TCR crosslinking is consistent with the hypothesis that immune activation may activate expression of latent HTLV-1, thus contributing to HTLV-1 pathogenesis. Our observations are complementary to those demonstrating additional important interactions between T cell activation and HTLV-1. The HTLV-1 p12(I) protein induces NFAT activation through cytoplasmic calcium release mimicking T cell activation (Ding et al., 2002). Interestingly, in these studies, p12(I) expression cooperated with PMA treatment in activation of NFAT (Ding et al., 2002), suggesting that at least two HTLV-1 proteins (Tax and p12(I)) have the ability to cooperate with T cell activation stimuli to further alter gene expression in infected cells. The HTLV-1 p30II protein has recently been shown to selectively activate genes involved in T cell activation and thus may also contribute to viral gene expression and pathogenesis through effects on T cell activation and proliferation, as well as effects on viral gene expression (Michael et al., 2004). The p30II protein may also have an important role in the establishment of latency. Nicot et al. have shown that p30II expression retains the mRNA's encoding Tax and Rex in the nucleus of infected cells, thus inhibiting viral gene expression (Nicot et al., 2004). Thus, the p30II protein, as well as Tax and Rex, through their interactions with cellular proteins may be critical determinants of viral latency or gene expression. It will be of interest to examine the function of p30II in unstimulated and stimulated FS cells.

T cell receptor activation involves the activation of TCR-associated tyrosine kinases, followed by induction of several signaling pathways, including those involving Ras and Rho− family GTPases, inositol phospholipids, protein kinase C, and calcium activation. Employing an Lck-defective mutant of Jurkat T cells allowed us to demonstrate that the Lck tyrosine kinase plays an important role in transduction of the PHA signal for activation of HTLV-1 expression, but is not required for PMA-induced LTR activation. Reintroduction of Lck by cotransfection with the LTR and Tax restored PHA-induced coactivation of the HTLV-1 LTR in these cells. This experiment complements and extends that of Dumais et al. (2003) who showed a similar dependence of CD3/CD28 and prostaglandin E_2 activation of the LTR on Lck function and is consistent with more recent studies showing a strong activation of the HTLV-1 LTR by tyrosine phosphatase inhibition (Langlois et al., 2004). Oncogenically activated Ras also strongly cooperated with Tax in activation of the HTLV-1 LTR. PHA or PMA treatment had little further effect over that induced by Ras and Tax. Cotransfection of a dominant-negative H-ras resulted in a reduction of Tax activation itself, as well as in PHA or PMA plus Tax coactivation. These data suggest that activated Ras can potently cooperate with Tax and may contribute both to basal Tax activation as well as to the PHA/Tax or PMA/Tax signal transduction pathway. The presence of significant residual coactivation by the PHA and PMA, even in the presence of dominant-negative form of Ras, suggests that additional signal transduction pathways generated in response to PHA or PMA are also important in PHA/Tax or PMA/Tax coactivation. Furthermore, the marked coactivation of the HTLV-1 LTR by activated Ras and HTLV-1 Tax, independent of PHA or PMA treatment, suggests that other signaling pathways that use Ras may also activate HTLV-1 gene expression in vivo. Interestingly, H-Ras and Tax have been previously shown to cooperate in the oncogenic transformation of primary rodent fibroblasts (Pozzatti et al., 1990). This suggests that cooperation between these two oncogenes may play a role in the induction of ATL. Similarly, activated Lck has been shown to cooperate with Tax in inducing growth factor-independent growth of hematopoietic cells (Miyazaki et al., 1996).

The mechanisms by which T cell activation stimuli cooperate with Tax to activate HTLV-1 LTR transcription remain unclear. To date, we have been unable to dissociate the coactivation effects from the effects of Tax alone on LTR expression (Lin et al., in preparation), suggesting either that transcriptional regulators induced by PHA or PMA are also dependent on Tax in order to activate LTR expression or that Tax itself is a target of the T cell activation stimuli. It is interesting to note that PMA has been suggested to increase Tax phosphorylation (Fontes et al., 1993), providing one possible mechanism for the coactivation induced by Tax and this stimulus. It is important to note that the transfection assays that we have employed express both Tax and reporter gene expression under the control of the HTLV-1 LTR, mimicking the provirus in which Tax and other HTLV-1 genes are controlled by the LTR. Thus, an additional explanation could be the generation of a very strong positive feedback loop in which the low levels of LTR activation seen with PHA or PMA alone (1.1- to 1.5-fold activation) result in sufficient increases in Tax expression to generate a strong amplification of LTR-directed expression of both Tax and the reporter gene. In this scenario, it would also be difficult to definitely dissociate the effects of PHA or PMA on the LTR from those due to increasing Tax synthesis.

HTLV-1 Tax also activates cellular promoters in addition to the HTLV-1 LTR (reviewed in Azran et al., 2004), through effects on subsets of CREB/ATF-, SRE-, and NF-κB-dependent promoters. Our data demonstrated that PHA or PMA signals further enhanced HTLV-1 Tax activation of promoters containing SRE- or NF-κB binding sites. Tax activates c-*fos* transcription by interacting directly with the serum response factor, which binds the central CArG sequence of the serum response element (Fujii et al., 1992). Tax activation of the SRE requires binding of the ternary complex factors (TCF) to the Ets box adjacent to the CArG box and Tax also directly interacts with TCFs in vitro

(Shuh and Derse, 2000). The HTLV-1 LTR has recently been shown to have a functional serum response element at −120 (Wycuff et al., 2004), suggesting that this site may also participate in LTR coactivation. These data suggest that PHA/Tax or PMA/Tax may activate the HTLV-1 LTR and SRE containing promoters through similar mechanisms. The mechanisms of Tax-mediated NF-κB activation are complex (Jeang, 2001). Recent data suggest Tax mainly affects NF-κB signaling in the cytoplasm through association with, and activation of, the IκB kinase complex (Li and Gaynor, 2000; Sun et al., 2000). Tax has also been shown to bind to the NF-κB p50 and p52 subunits in the nucleus, potentially directly enhancing NF-κB DNA binding and target gene transcription in the nucleus. We do not yet know the level through which PMA or PHA combine with Tax to increase NF-κB-mediated transcription. PHA and PMA seem to exhibit the same relative effects in the presence or absence of Tax, suggesting that they may exert separate additive effects on NF-κB-dependent transcription. It is important to note that cooperative promoter activation is not a feature of all cellular promoters or even all NF-κB-regulated promoters. Tax and T cell activation stimuli do not cooperate to activate the thymidine kinase promoter nor do they coactivate the promoter of the *Bfl-1* gene (data not shown), an NF-κB target gene regulated through a complex enhanceosome mechanism (Edelstein et al., 2003).

The pathogenesis of HTLV-1-associated diseases is clearly complex. Only a small fraction of infected individuals will develop disease after variable clinical latency periods. It is likely that host and viral factors both play important roles in the process. Latently infected cells are present in the blood of infected patients (Höllsberg, 1999; Kinoshita et al., 1989), potentially mediated through transcriptional repression by methylation of proviral DNA (Saggioro et al., 1991; Takeda et al., 2004). The potent cytotoxic T cell (CTL) response against Tax epitopes indicates that some level of Tax expression is persistent in vivo, however, and Tax-expressing cells may be readily cleared from the blood [reviewed in (Bangham, 2003)]. On the other hand, the high levels of CTL against Tax expressing cells may also serve as a strong selective pressure against Tax expression, thereby potentially favoring maintenance of latent infection. Other laboratories have demonstrated that anti-CD2 antibodies (Guyot et al., 1997) and cellular stresses activate HTLV-1 transcription from infected cells (Andrews et al., 1995), and our studies, as well as those of Dumais et al. (2003), suggest that T cell activation could also serve to increase HTLV-1 gene expression in vivo. This could result in increased levels of Tax in infected T cells, enhancing proliferation of the infected cells, through the effects of Tax-induced cellular proteins. Tax expression, which may induce either pro- or anti-apoptotic activities (Höllsberg, 1999), could also serve to overcome T cell apoptosis due to activation-induced cell death (AICD). Thus, we hypothesize that activation of Tax expression through T cell receptor stimulation would lead to increased T cell proliferation and inhibition of AICD, contributing to the oligoclonal T cell expansions seen in both pre-ATL as well as in HAM/TSP. This is similar to the model proposed by Azrin et al., in which cellular stresses activate HTLV-1 Tax expression and induce DNA damage and mutations, while induced Tax in turn blocks induction of cell death by these stimuli (Azran et al., 2004; Torgeman et al., 2001a). Furthermore, in the setting of T cell activation, the nature of the specific antigen-TCR interactions may lead to proliferation of subsets of T cells that may contribute to pathogenesis. It is also possible that such stimulation could increase HTLV-1 virus production, leading to spread of HTLV-1 within the infected individual to previously uninfected T cells. Some on-going replication occurs in vivo, as evidenced by the ability of reverse transcriptase inhibitors to decrease proviral load (Taylor et al., 1999). Most studies, however, demonstrate that increased proviral load is the result of expansion of already infected cells (Cavrois et al., 1998; Mortreux et al., 2003). Interestingly, chronic infection with *Strongyloides stercoralis* (Ss) results in a significantly higher HTLV-1 provirus load and an oligoclonal expansion of CD4+25+ T cells in dually infected, HTLV-1-positive individuals, associated with shortened clinical latency for the development of ATL (Gabet et al., 2000). Furthermore, Ss antigens may enhance T cell proliferation through activation of the IL-2/IL-2 receptor (Satoh et al., 2002). These observations parallel our hypothesis that T cell activation may enhance expression of latent HTLV-1 and contribute to the development of HTLV-1-associated disease phenotypes. Clearly further studies of the association of T cell activation and HTLV-1 activation in vivo will be required to further validate this hypothesis and to provide greater understanding of the relationship between HTLV-1 latency, viral activation, and disease pathogenesis.

Materials and methods

Cell lines

CDw32 L (Human Fc gamma RII receptor expressing mouse L TK-cells) (Peltz et al., 1988), obtained from the American Type Culture Collection, Rockville, MD, USA, on the behalf of DNAX Research Institute (Palo Alto, CA), were maintained in DMEM media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Product, Woodland, CA). Jurkat T cells, as well as the derived mutants, CD3-defective J.RT3 cells (ATCC, TIB-153) (Ohashi et al., 1985), and Lck-mutated JCaM.1 cell (ATCC, CRL-2603) (Straus and Weiss, 1992), were maintained in RPMI 1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% FBS. FS cells (Dezzutti et al., 1993), an HTLV-1-infected T cell line derived from a HAM/TSP patient, were maintained in RPMI 1640 medium containing 15% FBS and 50 U/ml

recombinant human interleukin-2 (IL2; Roche Applied Science, Indianapolis, IN).

Plasmids

pHTLV-1 Luc plasmid, an HTLV-1 LTR-luciferase reporter plasmid, has been described previously (Lin et al., 1998). pHTLV-1 Tax (Nerenberg et al., 1987), a Tax expression vector directed by the HTLV-1 LTR, was provided by Dr. Susan Marriott (Baylor University, Houston, TX). pcDNA-Lck, a plasmid expressing Lck protein under the control of the CMV promoter, was provided by Dr. S. Bour (NIAID, NIH, Bethesda, MD). pCMV-vRas, a plasmid expressing v-H-Ras under the control of the CMV promoter, was provided by Dr. C. Gélinas (UMDNJ-RWJMS, Piscataway, NJ). Other plasmids used included pMZH17N, expressing a human dominant-negative form of H-Ras (Asn17; Feig and Cooper, 1988) under the control of the CMV promoter (constructed by Dr. M. Malumbres and a kind gift of Dr. A. Pellicer, NYU Medical Center, New York, NY); pIL6-κB; a reporter plasmid containing three copies of the IL6-κB motif directing expression of the luciferase gene (Suh et al., 2002); pSRE-Luc, a reporter plasmid containing five repeats of the serum-responsive element (SRE) directing expression of luciferase (Stratagene, La Jolla, CA); and pRL-TK, a herpes simplex virus thymidine kinase (HSV-TK) promoter directing expression of the Renilla luciferase reporter gene (Promega, Madison, WI).

T cell activation and Western blot analysis

In the first approach for T cell activation, FS cells were cocultivated with irradiated (70 Gray) CDw32 L cells as described (Planken et al., 1996), which were pre-incubated with OKT3 antisera (murine OKT3, 5 μg/ml; Ortho Diagnostic System, Raritan, NJ) for 48 h. In the second approach for T cell activation, FS cells were incubated with MACSiBead particles coated with antibodies against human CD2, CD3, and CD28 (T Cell Activation Kit, Miltenyi Biotec., Auburn, CA) according manufacturer's instructions. 2.5×10^6 of FS cells were incubated with anti-Biotin MACSiBead particles (loaded with equal amounts of biotinylated antibodies against CD2, CD3, and CD28), at the ratio of 2:1 for 48 h. Total protein lysates were prepared by lysis of 10^6 cells in 250 μl of radioimmunoprecipitation assay (RIPA) solution (10 mM Tris [pH 8.0], 10 mM EDTA [pH 8.0], 100 mM NaCl, 1% Nonidet P-40, 0.25 mg of aprotonin per ml, 2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.5% sodium dodecyl sulfate [SDS]). Protein concentrations were determined by the BCA Protein Assay Reagent (Pierce Biotechnology, Rockford, IL). Twenty-five micrograms of total protein was electrophoresed through SDS–12.5% polyacrylamide gels and transferred to nitrocellulose filters (Protran BA85, Schleicher and Schuell BioScience, Keene, NH) with an electrotransfer apparatus. The filter was blocked by pre-incubation with 5% skim milk in phosphate-buffered saline–Tween 20 and incubated with Tax antibody (1:100 dilution, HTLV-1 Tax hybridoma 168A51-2, AIDS Research and Reference Program, Bethesda, MD). Control actin antibody (Catalog # A2066, Sigma-Aldrich, St. Louis, MO) was used at a 1:500 dilution. Antibody binding was detected with the ECL detection kit (Amersham Biosciences, Piscataway, NJ).

Transfection and luciferase assay

Reporter plasmids (5 μg), with or without pHTLV-1 Tax (1 μg), were introduced into T cells (10^7 cells per transfection) by DEAE-dextran transfection (Dorsett et al., 1983). Sixteen hours after transfection, cells were treated with Phytohemagglutinin (PHA-P, L9132, 5 μg/ml, Sigma-Aldrich, St. Louis, MO), phorbol 12-myristate 13-acetate (PMA, P1585, 50 ng/ml, Sigma-Aldrich, St. Louis, MO), or incubated with MACSiBead particles coated with antibodies against human CD2, CD3, and CD28 (T Cell Activation Kit, Miltenyi Biotec., Auburn, CA) according manufacturer's instructions. Cells were lysed and assayed for luciferase activity at 40 h after transfection as described by Luciferase Assay system (Promega, E4531, Madison, WI). Protein concentrations were detected by the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Luciferase activity was normalized to protein concentration (presented as activity per 20 μg cellular protein). Average luciferase activities were determined for triplicate transfections and standard deviations indicated by the error bars.

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